Sub-acute toxicity of licorice-sargassum extract in Sprague-Dawley rats: biochemical, histopathological, and pharmacokinetic studies

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To the Editor: The "eighteen antagonistic medicaments," an indispensable component of Oriental Pharmacology, states that sargassum and licorice should not be administered simultaneously.[1] Researchers have reported that co-administration of licorice and sargassum exerted toxic effects on the heart, liver, and kidney of rats.[2,3] However, the classic recipe "Hai-Zao-Yu-Hu-Tang," (containing licorice and sargassum) is currently used, though the safety profile of this medication has not been clearly defined. Therefore, a toxicological study of the combination of sargassum and licorice is necessary to determine its safety profile. In this study, a sub-acute toxicity test was used to evaluate toxicity. The main active components of licorice were liquirtin, isoliquiritin, liquiritigenin, isoliquiritigenin, and glycyrrhizic acid (GL). GL may be converted into glycyrrhetinic acid (GA) *in vivo*. Research has shown that the plasma concentrations of GA in rats are elevated after the oral administration of laminaria-licorice extract *vs.* licorice extract alone.[4] In this study, we developed an ultra-performance liquid chromatography coupled with triple-quadrupole mass spectrometry (UPLC-TQ/MS) method for the simultaneous determination of the six main components of licorice in rat plasma.

Sprague-Dawley rats were randomly divided into seven groups to orally receive normal saline (control), low-dose sargassum extract (2.66 g/kg) (LS), low-dose licorice extract (2.42 g/kg) (LL), low-dose licorice-sargassum extract (4.37 g/kg) (LLS), high-dose sargassum extract (5.33 g/kg) (HS), high-dose licorice extract (4.83 g/kg) (HL), or high-dose licorice-sargassum extract (8.75 g/kg) (HLS) twice daily for 4 weeks. Subsequently, blood samples were collected through the abdominal aorta to obtain serum for the analysis of biochemical indices. The heart, liver, and kidney were weighed and organ coefficients were calculated. Staining with hematoxylin-eosin was used for histopathological examination. The inflammatory response was specifically examined using CD68 immunohistochemistry staining. ACQUITY^{TM} Ultra Performance Liquid Chromatography (UPLC) and Xevo TQ/MS (Waters Corporation, Milford, Taunton, Massachusetts, USA) were used to detect the compounds. All data were acquired and processed using the Masslynx4.1 software (Waters Corporation). Quantification was performed by multiple reaction monitoring.

For the single-dose study, 12 Sprague-Dawley rats were randomly divided into two groups to orally receive licorice extract (4.83 g/kg) or licorice-sargassum extract (8.75 g/kg) on day 1. Blood samples (0.3 mL) were collected from the orbital vein at 0, 0.083, 0.25, 0.5, 1, 2, 3, 5, 8, 12, 18, 24, 36, and 48 h post-dosing. Plasma samples were also obtained. From day 3, the same rats continuously received their original daily dosage for another 7 days. For the multi-dose study, blood samples were obtained on day 9 as described above.

The organ coefficients [Supplementary Table 1, http://links.lww.com/CM9/A732] of the heart, liver, and kidney were significantly higher in the HLS group *vs.* the control group. In addition, the organ coefficients of the liver in both the HL and LLS groups were perceptibly increased compared with those obtained for the control group. There were no malformations nor color changes found in other organs. This finding suggested that the licorice-sargassum extract had a marked impact on the organ coefficients of the heart, liver, and kidney.

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As shown in Supplementary Figure 1, http://links.lww.com/CM9/A732 rats displayed a distinct elevation in serum biochemical indicators after co-administration of sargassum and licorice. The levels of creatine kinase in the HLS group and those of hydroxybutyrate dehydrogenase, lactate dehydrogenase (LDH), and aspartate aminotransferase (AST) in both the LLS and HLS groups were significantly higher than those measured in the control group, indicating that the licorice-sargassum extract exerted a toxic effect on the heart. The levels of alanine transaminase in the HLS group, and those of AST and alkaline phosphatase in both the LLS and HLS groups, were markedly increased compared with those detected in the control group, suggesting that the licorice-sargassum extract was toxic to the liver. The levels of blood urea nitrogen in LL, HL, and HLS groups, and those of creatinine in the HL group, were significantly higher than the levels recorded in the control group, implying that the licorice and HLS exerted a toxic effect on the kidney. Furthermore, the levels of LDH in the LLS and HLS groups, as well as those of triglycerides in the HS, LLS, and HLS groups, and glucose (GLU) in the HL and HLS groups, were perceptibly higher than the levels observed in the control group. These findings suggested that the combination of licorice and sargassum would probably cause GLU and lipid metabolism disorder in the body.

As shown in Figure 1A and Supplementary Table 2, http://links.lww.com/CM9/A732, a severe pathological response in cardiac tissues was present in the HLS group at the end of 4 weeks. Inflammation was the main pathological feature. The LS group demonstrated a medium-grade inflammatory cell infiltration and degeneration of the myocardium. Limited infiltration of inflammatory cells was presented in the HS group. Both LLS and HLS groups demonstrated inflammatory cell infiltration accompanied by inflammatory exudation at the edge of the heart tissue. Neutrophil granulocyte infiltration and some destroyed myocardial cells were observed in the HLS group. Figure 1A and Supplementary Table 2, http://links.lww.com/CM9/A732 show the pathological change in the liver. The LL group showed inflammatory cell infiltration around the central vein. The HL group showed a mild inflammatory response and a small amount of cell edema. The LLS group demonstrated a moderate lymphatic infiltration of the bile duct. The HLS group showed lymphatic infiltration of the bile duct and liver cell degeneration, suggesting cardiogenic liver cirrhosis. The primary pathological change in the kidney was an inflammatory response. The HS and HL groups observed moderate inflammatory cell infiltration. The LLS group showed pyelonephritis and inflammatory infiltration around the renal tubules. The HLS group showed thickening of the wall epithelium, proximal tubules, and renal tubules, and infiltration of inflammatory cells around the renal tubules, accompanied by pyelonephritis. A significant increase in CD68+ macrophages in HLS group [Figure 1B] was observed compared with the other groups, indicating an increase in infiltrating monocytes or Kupffer cell activation and enhanced inflammation in cardiac, hepatic, and renal injury.

In compand detection, there was no endogenous interference in the sample [Supplementary Figure 2, http://links.lww.com/CM9/A732].

The UPLC-TQ/MS method, developed to quantitative compounds in pharmacokinetic studies, was validated by the linearity, lower limits of detection and quantification, intra- and inter-day precisions, extraction recovery, matrix effect, repeatability, and stability [Supplementary Tables 3–6, http://links.lww.com/CM9/A732]. In Supplementary Figure 3A and Supplementary Table 7, http://links.lww.com/CM9/A732, the area under curve (AUC), C_{max} and T_{max} of liquirtin, isoliquiritin, liquiritigenin, and isoliquiritigenin were decreased after the administration of a single-dose of the licorice-sargassum extract compared with licorice extract. This was consistent with the results obtained in the multidose administration experiment [Supplementary Figure 3B and Supplementary Table 7, http://links.lww.com/CM9/A732]. Considering that these four compounds belong to the flavonoid family, it is possible that the accumulation of GL hindered the absorption of these components in the body. However, the levels of liquirtin, isoliquiritin, liquiritigenin, and isoliquiritigenin in the licorice extract were low, indicating that these compounds may not contribute to the toxicity of the licorice-sargassum complex. Administration of a single-dose of the licorice-sargassum extract increased the AUC and C_{max} of GL and decreased the half-life ($T_{1/2}$). However, following the administration of multiple doses of the licorice-sargassum extract, the AUC of GL was slightly decreased. It is possible that the cumulative addition of GA inhibited the secondary absorption of GL *in vivo*. The double-peak phenomenon displayed by GL and GA may be explained by the hepatoenteral circulation of licorice. Single- and multi-dose administration of the licorice-sargassum extract significantly increased the AUC and C_{max} of GA.

Sargassum and licorice are both widely used in food, health products, and drugs. The "Hai Zao Yu Hu Tang" recipe, which contains licorice and sargassum, is currently used for the clinical treatment of thyroid tumors and breast hyperplasia. It is possible that different proportions of licorice and sargassum determine the therapeutic effect and toxicity of this treatment.[2,3] However, toxicological studies are lacking, and the mechanism involved in the combination of sargassum and licorice remains unclear. In our study, we found that the licorice-sargassum extract was toxic to the heart, liver, and kidney in rats. Studies have revealed that GL is the main component of licorice, which is metabolized in the intestine and ultimately affects the body in the form of GA.[5] Excessive levels of GA can lead to false aldosteronism since its chemical structure is similar to that of corticosteroids.[6] Pharmacokinetic studies in rats showed that the licorice-sargassum extract significantly increased the plasma concentration of GA compared with oral administration of licorice extract only. This was consistent with the increase in plasma GA after co-administration of licorice and laminaria extract.[4] In view of the chemical similarity of laminaria and sargassum, we hypothesized that the toxicity of the licorice-sargassum combination may be due to increased levels of GA resulting from an increase in the concentration of GL in plasma.

In summary, the toxic effects of the licorice–sargassum extract targeted the heart, liver, and kidney. This toxicity may result from sargassum promoting the absorption of GA in licorice. Further study is warranted to provide

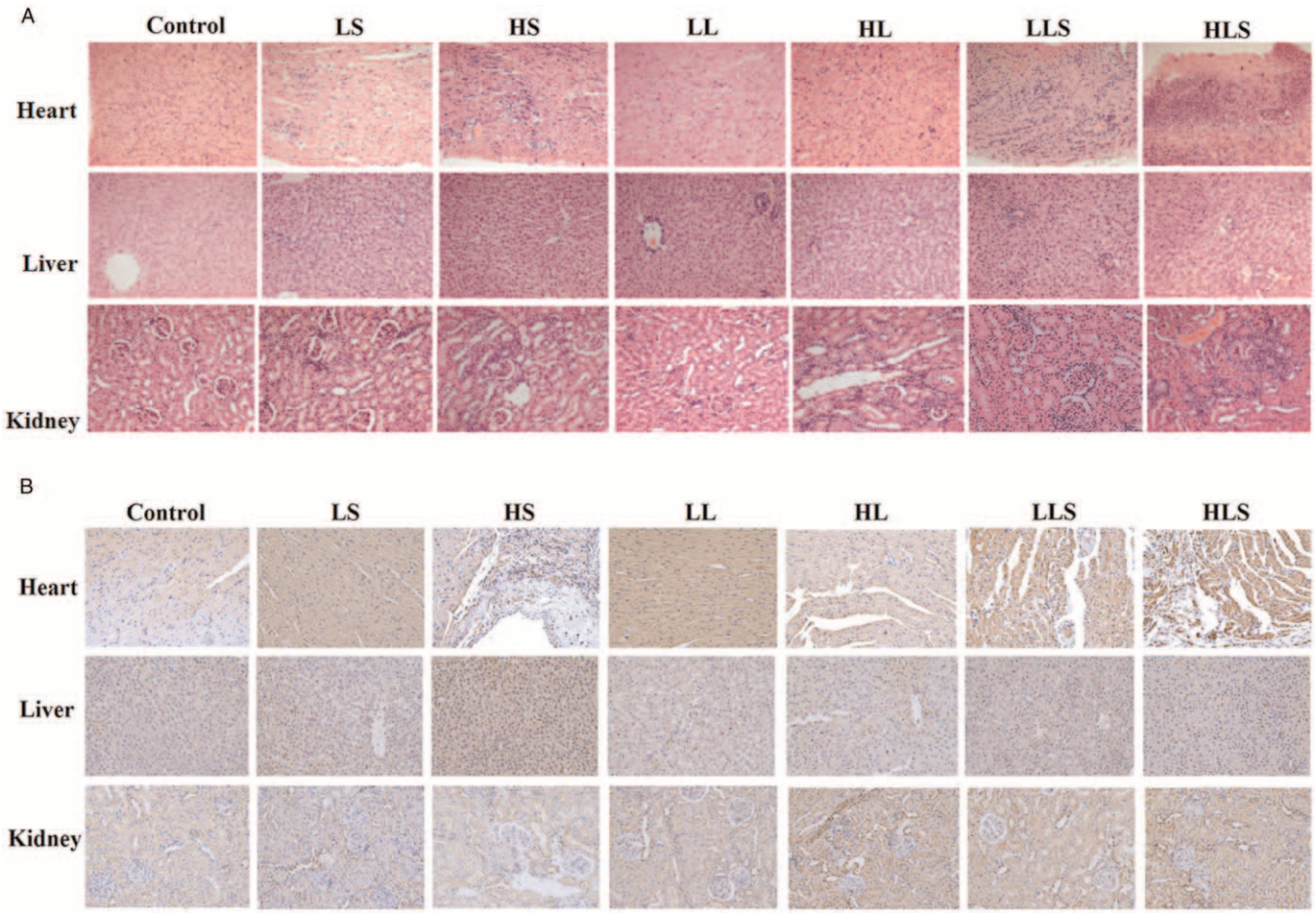

Figure 1: Co-administration of licorice and sargassum showed toxic effects in rats. (A) Histopathology of heart, liver and kidney In all groups. (B) CD68 Immunohistochemistry staining of heart, liver, and kidney in all groups. GA: Glycyrrhetinic acid; GL: Glycyrrhizic acid; HS: High-dose sargassum extract; HL: High-dose licorice extract; HLS: High-dose licorice–sargassum extract; LS: Low-dose sargassum extract; LL: Low-dose licorice extract; LLS: Low-dose licorice-sargassum extract.

guidance for the combined use of licorice and sargassum in clinical practice, and may also reveal the chemical basis of the licorice-sargassum complex.

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Conflicts of interest

None.

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